An In Silico Approach to Analyze Epicatechin Gallate and its Derivatives as Effective Antifibrotic Agents during Wound Healing

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By
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CERTIFICATE

This is to certify that the thesis entitled **" An *In Silico* approach to analyze Epicatechin Gallate and its derivatives as Effective Antifibrotic Agents During Wound Healing"** by **Dhruva kumar r (210bm2012)** submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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ABBREVIATIONS

PDB: Protein data bank

MDS: Molecular dynamic simulation

RMSD: Root mean square deviation

TGF: Transforming growth factor

TRP: Tryptophan

K.E: Kinetic energy

P.E: Potential energy

T.E: Total energy

Asp: Aspartic acid

Thr: Threonine

Phe: Phenylalanine

Lys: Lysine

Gln: Glutamine

His: Histidine

Arg: Arginine

ECG: Epicatechin Gallate

ADME: Absorption Distribution Metabolism Excretion

BMP: Bone morphogenetic protein

ALK: Anaplastic lymphoma kinase

ABSTRACT

The prospering wound healing treatment is one that minimizes the formation of connective tissue and reduces scar that is produced during healing process. Scar formation in healing can be reduced by blocking the prominent pathway responsible for it. Previous studies identify TGF-β as the major player in the pathways that lead to scar formation and thus can be targeted for exploring Antifibrotic drugs. The objective of the current study is to search and validate an effective inhibitor of TGF-β receptor to prevent scar formation during wound healing by utilizing the computational methods. Briefly, a group of newly discovered molecules from Drug Bank and other natural compounds predicted to act in TGF β pathway were selected to check their inhibitory activity against TGF-β1 receptor. The ligands were docked against the active site of TGF-βR1 in Autodock4. A natural compound, Epicatechin Gallate showed highest binding energy (-9.44 kcal/mol) compared to the control, SB505124 (-9.18 kcal/mol). Since previous studies have confirmed that Epicatechin Gallate is only effective in minor scars, design of an effective inhibitor for extensive scar was attempted by modifying the parent structure of Epicatechin Gallate. A series of ligands thus obtained exhibited better binding energy upon docking. Two such derived ligands showed a binding energy above -10.4kcal/mol. However, one of these derived ligands with added hydrophobicity at C5 position qualified for Drug likeness, Toxicity and ADME properties in PreADMET server. The same ligand also made a stable complex with the target as obtained in terms of RMSD and Total Energy measures by real time Molecular Dynamic Simulation studies. All the studies indicate S1 can act as effective inhibitor of TGF-β pathway. But further clinical trials were necessary to confirm its action.

Key Words: TGF-β, Drug bank, Docking, Molecular Dynamic Simulation, ADME, PreADMET, RMSD, Autodock.

2. INTRODUCTION

2.1 Wound

A wound occurs when the wholeness of any tissue is negotiated. A wound may be induced as a result of a cut or by an infectious disease; [1].

2.2 Types of wound

Acute wounds are categorised in to these many types by emergency personnel and first aid workers.

1. Abrasions (scrapes) – skin scratched away due to frictional force.
2. Avulsions – constrained removal of tissue from body.
3. Lacerations – blunt and irregular breaks due to use of high force.
4. Punctures – narrow and deep wound due to penetration of sharp objects like a pin.
5. Incisions – sharp cut due to sharp instrument.
6. Contusions – internal injury due to forced trauma.

2.3 Wound healing

Wound healing, or wound repair, is an intricate process in which the skin repairs itself after injury. The successful wound treatment is one that reduces the formation of scar tissue and decreases the amount of necrotic tissue that is produced during this process [2].

2.3.1 Normal Wound-Healing Phases.

The wound passes through 4 phases so as to impact a final repair

1) Barrier protection
2) Inflammatory phase,
3) Fibro plastic phase and
4) Remodelling phase.

2.3.1.1 The physical barrier

Unless preventive measures are taken wounds will readily acquire bacteria. Established absorbent cellulose affords limited protection against bacteria, particularly in the presence of serous exudate that may negotiate dressing integrity. In addition, particles remaining in wound are shed by dressings. Many advanced dressings are impermeable to bacteria, are removed completely, and have been found to reduce the incidence of wound sepsis and optimize re epithelialization rates.

2.3.1.2 The inflammatory phase

The inflammatory phase sets up the area for healing and traps the wound by causing it to swell and develop pain, so that restricting the movement. The fibroplastic phase reconstructs the structure, and then final form is produced by remodelling phase. Inflammation is a essential prerequisite to healing.

2.3.1.3 Fibroblastic Phase

Rebuilding can commence after completion of inflammatory phase. It is named for the principal cell of scar production - the fibroblast. There are fewer types of cells operate and finishes about three weeks. During this phase the wound is resurfaced and passed on with

strength. The fibroblasts are originated from mesenchymal cells which are located in loose tissue around blood vessels and fat.

2.3.1.4 Remodelling *Phase*

***Epithelialisation*.**

Blood flow, phagocytosis, are the ingredients critical to tissue persistence, and the provision of a surface covering happen early in the healing process. The preparation of even a one-cell layer will furnish protection from external invading organisms. Within hours after injury, undamaged epithelial cells begin to reproduce. Epithelial mitosis is regeneration process which leads to a ridge forming around the periphery of the wound.

Wound contraction.

Epithelialisation closes wound surface, but contraction pulls the entire wound together in effect quenching the defect. A effective contraction results in a smaller wound to be repaired by scar formation. Decreasing the area to be healed is truly beneficial in certain tissues with fixed, deep structures covered by mobile, loose skin.

Collagen production.

Wound healing is terminated by collagen production which is essential if wound healing is to occur. Collagen is synthesised by the migratory fibroblasts present throughout the wound. Collagen produced is influenced by development of lactic acid.

Collagen fibre orientation

Scar tissue is haphazardly deposited in remodelling phase to be arranged, in both lateral and linear orientation. Scar tissue being non-flexible and tries to mimic the characteristics of the tissue in which it is healing. Collagen weave is induced by tissue structure, thus dense tissues

induce highly cross-linked scar; pliable tissues induce a loose, coiled, less cross-linked scar. This process does not function as expected and results in unwanted scar tissue in the form of hypertrophic or keloid scars[1, 2].

Figure1. Phases of wound healing for, Haemostasis (a), Inflammatory (b Proliferative(c), Remodelling (d)

2.4 SCAR FORMATION

Cutaneous wound healing is a complicated process, which involves many different aspects including growth factors, extracellular matrices and various cell types [3]. This healing process in adult human being or adult animals of higher vertebrate is usually overactive and can cause scar formation. Scarring is an unpleasant lesion to patients physically and psychologically. Sometimes, it also causes functional disability in severe burn patients. Keloid is an example of aberrant scarring in human being, which usually extends beyond the boundary of an original wound and is difficult to treat clinically. Fortunately, the scar less wound healing process in fetal wound repair may provide insights into the potential

conversion of adult wound healing into a healing process similar to that of fetal scar-free wound. The development of gene therapy techniques offers a powerful tool to genetically modify the healing process of adult wounds to a repair process similar to fetal wounds. Because of the close relationship between sustained expression of transforming growth factor- β (TGF- β) and the formation of keloid and hypertrophic scar as well as transient, lower level expression of TGF- β in fetal scar less wound healing this article intends to review the roles of TGF- β in scar formation, the strategies of antagonizing wound TGF- β and preliminary results of scar gene therapy by targeting wound TGF- β .

2.5 Mechanisms of TGFβ- Signalling from Cell Membrane to the Nucleus

TGFβ is initiated by type I and type II receptor serine/theorine kinases on the cell surface. Phosphorylation of type II receptor propagates signal through phosphorylation of Smad proteins. Type I receptor activates Co-mediator Smad (Co-Smad), the receptor-regulated Smad (R-Smad), and the inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5, and 8) by direct phosphorylated undergo homotrimerization and formation of heteromeric complexes with the CoSmad, Smad4 , R-Smad. Which are translocated into the nucleus along with co factors and regulate the transcription of target genes. The I-Smad, Smad6 and Smad7, negatively regulate TGFβ- signaling by engaging with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation[4].

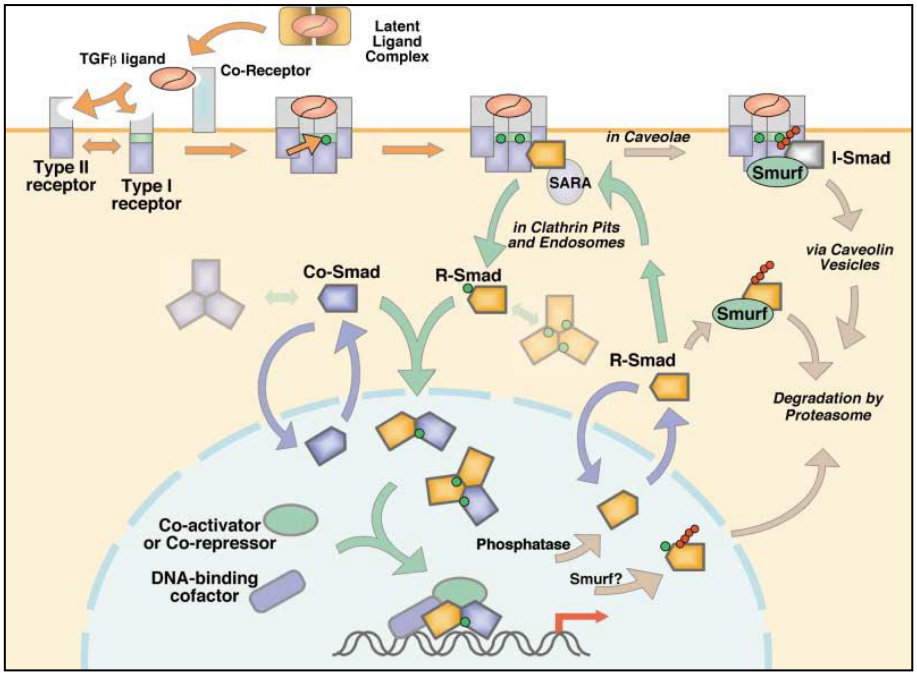


Figure 2 - Schematic Diagram of TGF-β Signaling from Cell Membrane to the Nucleus[4]

2.5.1 TGF-β Ligands and Receptors

The TGFβ- family of cytokines, , are encoded by 42 open reading frames in human, 9 in fly, and 6 in worm characterized by six conserved cysteine residues [5]. The ligands dimeric arrangement suggests the formation of a complex with two type I and two type II receptors. Family of proteins collectively known as Ligand traps regulate the ligand access to the receptor. The receptor serine/threonine kinase family comprises 12 members—7 type I and 5 type II receptors—all dedicated to TGFβ- signalling in human genome [6]. Both types of the receptor serine/threonine kinases consist of about 500 amino acids, organized sequentially into an N-terminal extra-cellular ligand binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain. Three-finger toxin fold is exhibited by the overall structures of the extracellular ligand binding domain of the type I BMP receptor [7] as well as the type II receptors for Activin [8] and TGFβ [9], with each finger formed by a pair of anti-parallel strands.

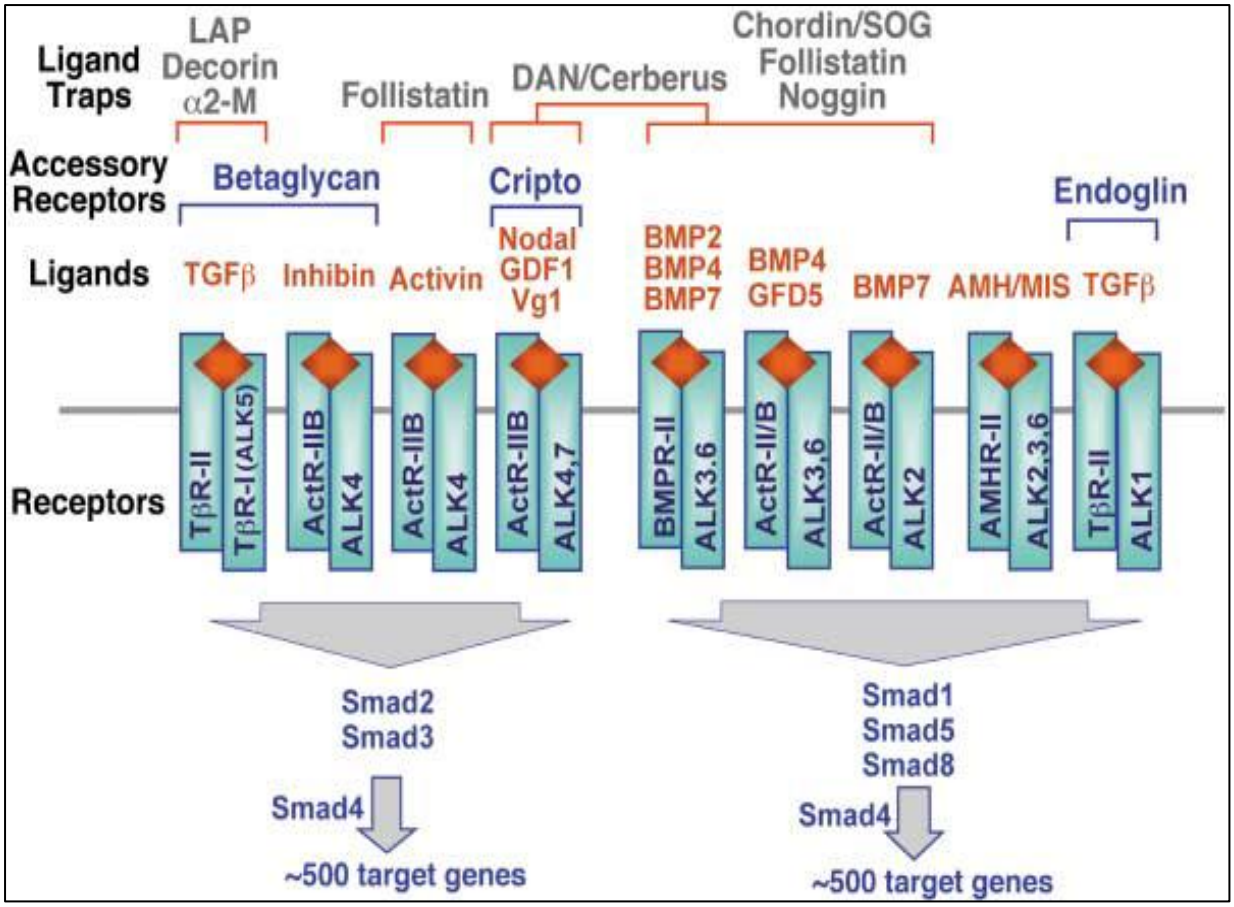


Figure 3 - A Formal Relationship Depicting TGF-β Ligands, , Type I and II Receptors ,Accessory Receptors, Ligand Binding Traps in Vertebrates[4]

3. OBJECTIVE

- To explore the agents that are linked to TGF-β and it's receptors, central to transduction pathways responsible for scar formation
- To identify a best drug amongst existing drug bank inhibitors of TGF-βR1 by binding energy estimation from ligand-protein docking
- To design synthetic drugs from the best inhibitor by structural modification *in Marvin Sketch*.
- To analyze the suitability of the novel inhibitors as drugs in Pre ADMET server.
- To validate the stability of ligand-protein complexes by Molecular Dynamics Simulation studies.

4. LITERATURE REVIEW

4.1 TGFβ AND WOUND SCARRING

In contrast to fetal wounds, enhanced expression of TGFβ and its receptors has been observed in hypertrophic scar and keloids. The hypertrophic scar fibroblasts expressed TGF- β1 mRNA in a level significantly higher than that of the normal fibroblasts. Schmid et al. found TGF- β receptors I and II expressing more in granulations tissue than in the normal skin at both mRNA and protein levels[10]. Similarly, Lee et al. have shown in an in vitro study that keloid fibroblasts produced more TGF- β1 and TGF- β2 protein, but not TGF- β3, than normal fibroblast[11]. Chin et al. further demonstrated an increased protein expression of TGF- β receptors I and II in the cultured keloid fibroblasts as compared with the normal dermal fibroblasts[12]. These results suggest that overexpressed TGF- β ligands and their

receptors are associated with scar and keloid formation. Hence by targeting wound TGFβ wound scarring can be significantly reduced[13].

4.2 CELLULAR AND MOLECULAR MECHANISMS OF TGF-β IN SCAR FORMATION

Although the mechanism of scar formation involves many different aspects and is too complicated , a number of studies have shown that TGF- β plays a key role in scar formation via different mechanisms such as inflammation, matrix production, matrix remodeling, and the regulation of cell proliferation and apoptosis, etc. During wounding, the initial dose of TGF- β appears to be an important factor to trigger the later cascade of TGF- β overproduction and scar formation. When a wound is created, TGF- β released from the platelets can recruit monocytes and other cells into the wound, and these cells further produce more TGF- β in the wound. Additionally, the initial dose of TGF- β in the early wound induces fibroblasts, the key player of scar formation, to produce their own TGF- β in the later stage wound via autocrine regulation, leading to the overproduction of wound TGF- β and eventually to scar formation . In vitro studies have shown that TGF- β 1 promoted cell proliferation and collagen production of both keloid and normal dermal fibroblast, and the collagen production stimulated by TGF- β was mediated by Smad3 and Smad4 [14]. In vivo, overexpression of TGF- β1 gene has been observed at the peripheral area of keloid tissues, where highly proliferated cells reside. In addition, TGF-β1 gene expression is also co-localized to the area where collagen gene is actively expressed; suggesting that TGF- β 1 produced by keloid fibroblasts also promotes their proliferation and collagen production in the local environment of keloid tissues.Wound tension is also known to promote scar formation. In an in vitro assay, Peled et al. have shown that mechanical strain could up-regulate the gene expression of TGF- β 1 and

its receptors as well as type I procollagen in normal dermal fibroblasts, indicating that enhanced expression of TGF- β 1 and its receptors by cellular strain may contribute to the tension-induced scarring

4.3 STRATEGIES OF BLOCKING TGF- β BIOLOGICAL ROLES IN WOUNDS

Wound scarring remains difficult to cure. Conventionally, scarring is considered as a natural result of wound healing and is inevitable. The study by Longaker *et al.* in a sheep model, however, demonstrated that the cleft lip defect of early gestational fetal sheep could be repaired without any scar formation[15]. This interesting phenomenon indicates that scar is not the necessary result of healing mammalian wounds, and adult wounds may be ideally repaired by tissue regeneration rather by fibrous tissue if their healing processes can be modified to mimic the mechanism of fetal scarless wound healing. A pioneer study by Shah *et al.* demonstrated for the first time neutralizing wound TGF- β with anti-TGF- β antibodies in adult rodent[16]. This study provides direct evidence that manipulation of TGF- β effects in adult wounds is a good strategy of reducing wound scarring. Importantly, local manipulation of wound TGF- β can avoid the adverse effect of systemic manipulation of TGF- β, which may cause a lethal effect on experimented animals[17].

Manipulation of wound TGF- β at early stage is essential to the reduction of scarring, because the initial dose of TGF- β can trigger the cascade of TGF- β overproduction in wounds via its chemotaxis and autoinduction, which stimulates fibroblast proliferation and collagen production, inhibiting degradation of matrix and leads to scar formation[18].

The second way of manipulating wound TGF- β effects at protein level is the use of TGF- β antagonists. Several different molecules are found to be able to bind TGF- β ligands and

block their biological effects. Decorin is a small chondroitin/dermatan sulfate proteoglycan [19]. The core protein fragment (Leu155-Val260) of decorin binds TGF- β[20] and inhibits TGF- β mediated biological effects[21, 22]. Border *et al.* injected decorin molecules intravenously and successfully inhibited the fibrotic process of glomerulonephritis, in which TGF- β also plays an important role in the pathogenesis[23]. Biglycan is another small proteoglycan that is able to bind TGF- β. Nevertheless, it may regulate TGF- β's effect *in vivo* differentially from decorin[24]. Soluble forms of TGF- β receptors I and III (betaglycan) have been found in different cell types as a result of natural process of mRNA splicing[25, 26]. Because the binding regions for TGF- β ligands are located at the extracellular domains, the soluble receptors remain able to bind their ligands but fail to signal intracellularly, and thus can serve as TGF- β antagonists. Since TGF- β receptor II initiates the ligand binding and the intracellular signaling, most studies used recombinant soluble TGF- β receptor II as an antagonist to neutralize various TGF- β effects[27-30].

The third approach is to block TGF- β activation. Natural TGF- β is secreted as a biologically latent form, which can be activated by transient acidification, proteolysis, and chaotropic agents [Brown *et al.,* 1990]. Since TGF- β can be activated via the binding of mannose-6-phosphate (M6P) residues on LAP to the M6P receptors on cell surface[31], excessive M6P injected into wounds can compete with LAP and inhibit the activation of wound. TGF- β, and thus reduce scarring[32]. An antibody to LTBP or to transglutaminase and transglutaminase inhibitor may also inhibit the activation of latent TGF- β[33].

4.4 Inhibitors of TGFβ pathway to reduce scar formation

J-A Moon et al showed (TGFβ) plays a primarily role in the progression of renal fibrosis. IN-1130, a new small molecule ALK5 inhibitor, inhibited the purified kinase domain of ALK5-mediated Smad3 phosphorylation with an IC50 value of 5.3 and proved to be majorly selective in a panel of 27 serine/threonine and tyrosine kinases [34].

Anne-Charlotte de Gouville etal GW6604 (2-phenyl-4-(3-pyridin-2-yl-1H-pyrazol-4-yl) pyridine), an ALK5 inhibitor, to assess the therapeutic potential of inhibiting TGFβ pathway in different models of liver disease. GW6604 blocks TGFβ effects in vivo and inhibits TGFβ signaling in vitro [35].

Rudy Ciayadi etal synthesised novel inhibitors of TGFβ1 based on a 2-aryl-4-(3-(pyridin-2-yl)-1H-pyrazol-4- yl) pyridine compounds containing phenyl or aromatic nitrogen heterocycle substituents. TGFβ1 is selectively inhibited in HEK-293T cells in culture [36].

Hiroyuki Higashiyama etal SB-525334 treatment attenuated significantly decreased the type I and III procollagen and fibronectin mRNA expression in the lung [37]. Dominique Bonafoux etal The 4-(5-fluoro-6-methyl-pyridin-2-yl)-5-quinoxalin-6-yl-1H-imidazol-2-ylamine 3 is a powerful and selective inhibitor of TGFβR1. Replacement of the amino group of 3 typically led to a slight decrease in the affinity for the receptor and in TGFβ-inducted PAI-luciferase reporter activity. However, 2-acetamidoimidazole was attractive candidate for further optimization as a result of their significant activity combined to their superior pharmacokinetic profile [38].

Sunil K. Halder etal investigated the potential of TRKI in new therapeutic approaches in preclinical models. Transcription, gene expression, apoptosis, and growth suppression of

TGF-β is induced by TRKI, SB-431542. SB-431542 was capable of rarefying TGFβ induced EMT the tumour-promoting effects of TGF-β, including cell motility, migration and invasion, and vascular endothelial GFs secretion in human cancer cell lines[39].

Chul-Yong Park etal examined a new small molecule inhibitor of ALK5, 3-((5-[1, 2, 4] triazolo [1,5-a]pyridin-6-yl)-4-(6-methylpyridin-2-yl) thiazol-2ylamino) methyl) benzonitrile (EW-7203) in breast cancer cells to check if it has potential for cancer treatment. The inhibitory effects of EW-7203 on TGFβ-induced Smad signalling and epithelial to-mesenchymal transition (EMT) were examinied in mammary epithelial cells using luciferase reporter assays, immunoblotting, and confocal microscopy and wound healing assays. The novel ALK5 inhibitor, EW-7203, expeditiously inhibited TGFβ1-induced Smad signalling [40].

Francüoise Gellibert etal identified a novel 1, 5-naphthyridine aminothiazole and pyrazole derivatives, has selective inhibitors of the transforming growth factor-*â* type I receptor, ALK5. James F. Callahan, etal suggested that suppression of ALK5 by GW6604 (2-phenyl-4-(3-pyridin-2-yl-1H-pyrazol-4-yl) pyridine), could be an appealing approach to treatment of liver fibrosis. Werner J[41]. Geldenhuys etal SB-505124 and A83-01 binding to the ATP Binding site of TGFβR1 and act as competitive inhibitors, and effective inhibition of TGFβ in cell culture[42] .

N. J. LAPING, etal expressed the kinase domain of the TGF-β type I receptor [Activin receptor-like kinase (ALK) 5] and the substrate, Smad3, and determined that SB- 431542 is a selective inhibitor of Smad3 phosphorylation with an IC50 of 94 nM. It inhibited TGF-β induced nuclear Smad3 localization. The p38 mitogen-activated protein kinase inhibitors SB-203580 and SB-202190 also inhibit phosphorylation of Smad3 by ALK5 with IC50 values of 6 and 3M, respectively brotic diseases by both avoiding matrix deposition and promoting

hepatocyte regeneration[43]. Kai Fu, Michael J etal SM16 blocked TGFβ-induced plasminogen activator inhibitor (PAI) luciferase activity in cells, TGF-β and Activin-induced Smad2/3 phosphorylation [44].

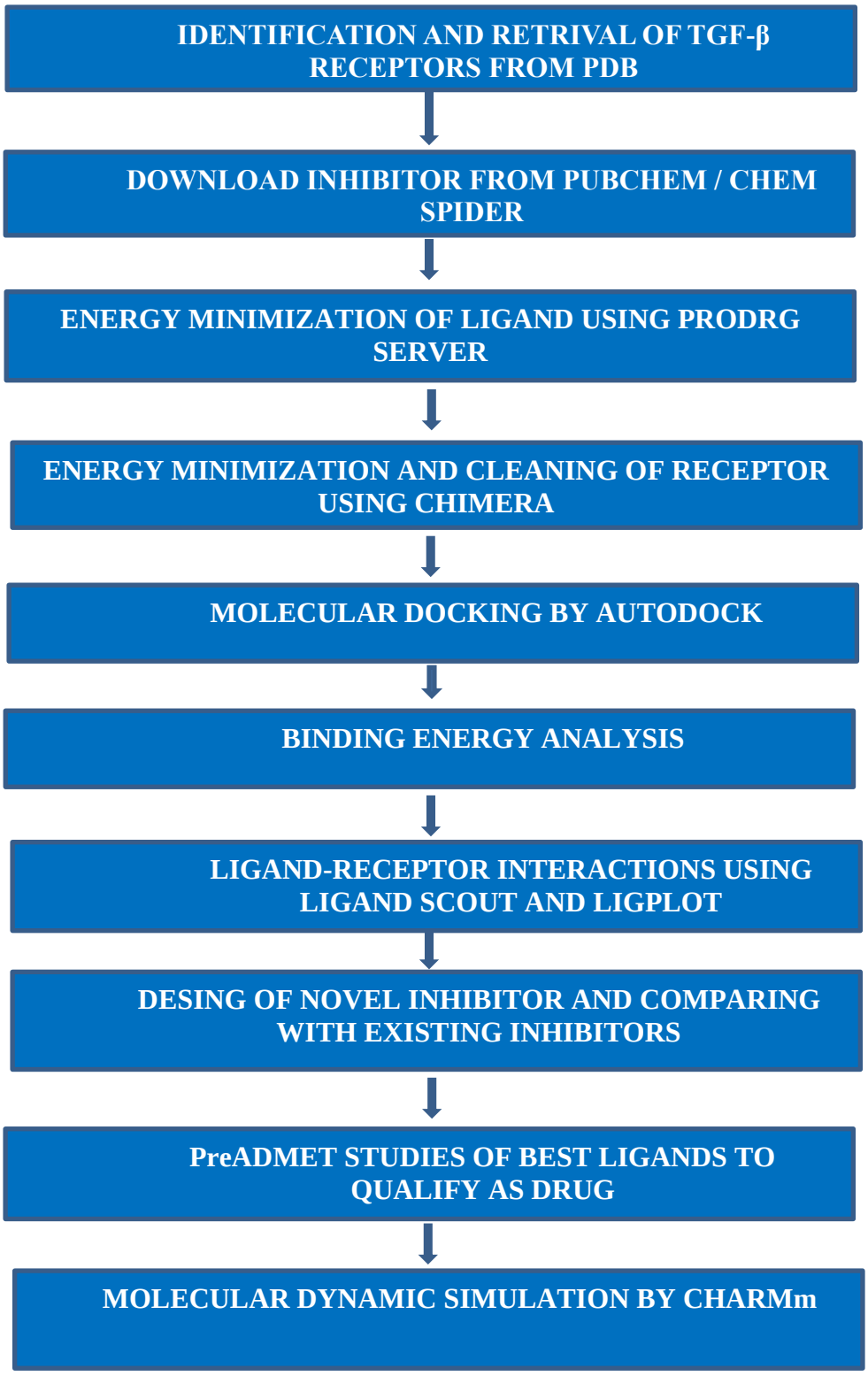
Stacey DaCosta Byfield etal In their study, characterized 2-(5βenzo [1,3]dioxol-5-yl-2-*tert*βutyl-3Himidazol-4-yl)-6-methylpyridine hydrochloride (SB-505124 which suppress the TGF-βR1 identified as ALK5. Reveal that this compound selectively and concentration-dependently inhibits ALK4, ALK5, and ALK 7 dependent activation of downstream cytoplasmic signal transducers, but does not alter ALK1, ALK2, ALK3 or ALK6-induced Smad signaling[45].

Mohit Kapoor etal inquired scar formation reduction effect of the ECG, in a full thickness incisional pattern of wound healing in rats. Significant improvement in the quality of scar formation both in terms of development and orientation of the collagen fibres was shown by ECG . For the first time, their study revealed that catechins, namely ECG, can significantly improve the quality of wound healing and scar formation [46].

Werner J.etal Compounds such as SB-505124 and A83-01 have been shown to bind to the ATP Binding site of TGFβR1 and act as competitive inhibitors, and in cell culture have shown effective inhibition of TGFβ. Structurally, most of these compounds, which have been published, share a similar central core consisting of a pyrazole or imidazole with a pyridine[47].

Chul-Yong Park etal inquired a novel small molecule inhibitor of ALK5, (EW7203) in breast cancer cells to identify if it has potential for cancer treatment. The novel ALK5 inhibitor, EW-7203, inhibited the TGFβ1-stimulated transcriptional activation of p3TP-Lux and pCAGA12- Luc. The novel ALK5 inhibitor, EW-7203, efficiently inhibited TGFβ1-induced Smad signalling.

5. Plan of work



6. MATERIALS AND METHOD

6.1 Tools used:

- **Marvin Sketch** - For drawing Novel Ligand/Inhibitor
- **Online Translator -** For Translating ligand file format to pdb
- **PDB -** For Downloading Receptor/Protein .pdb files
- **Pubchem** – Database of inhibitors
- **Prodrg Server-** Energy minimization of inhibitors
- **Chimera** - For Visualization and Energy Minimization studies
- **Pymol** – For visualization and alignment
- **Hex -** For Automated Molecular Docking (protein +protein)
- **Auto Dock 4** - Potential offline Docking(protein+ligand) tool widely used
- **Ligand scout** - To find the specific interactions
- **Lig plot –** To find out atomic interactions
- **PreADMET-** To check drug likeness
- **CHARMm -** Molecular dynamics simulation

6.2 Retrieving receptors from pdb:

- Open Protein data bank, pdb.org
- Type receptor name in search i.e TGFβ receptor.
- Retrieve the pdb files of the receptors and store them in pdb file format for further use.

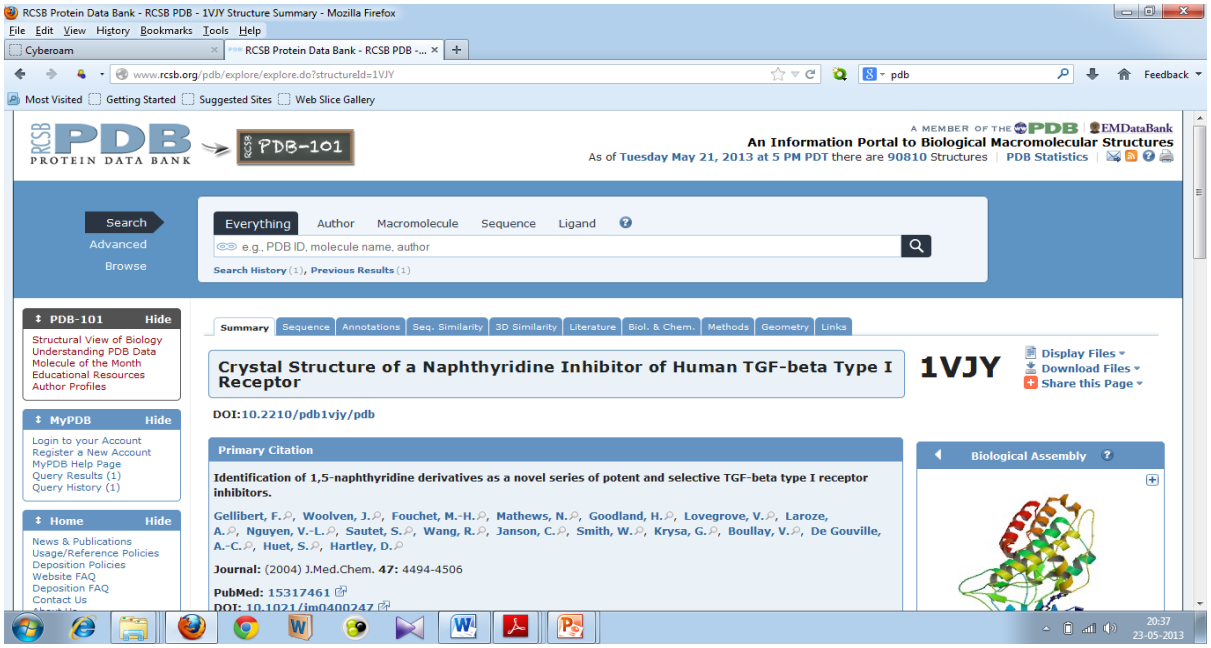


Figure 4 - Snapshot of Protein Data Bank webpage with TGFβ1receptor (PDB ID: 1VJY)

6.3 Retrieving ligands from Pubchem and conversion to pdb format by Marvin sketch:

- Open pubchem pubchem.ncbi.nlm.nih.gov
- In search type the name of inhibitor & enter
- All the available ligands will appear, if something is missing search for the same in chemspider.
- Save the 3D .sdf format of the ligands from pubchem and chemspider.
- Open the above .sdf format in Marvin sketch and save as .pdb
- Now the pdb format ligand is in 3D structure that will be used in following steps.

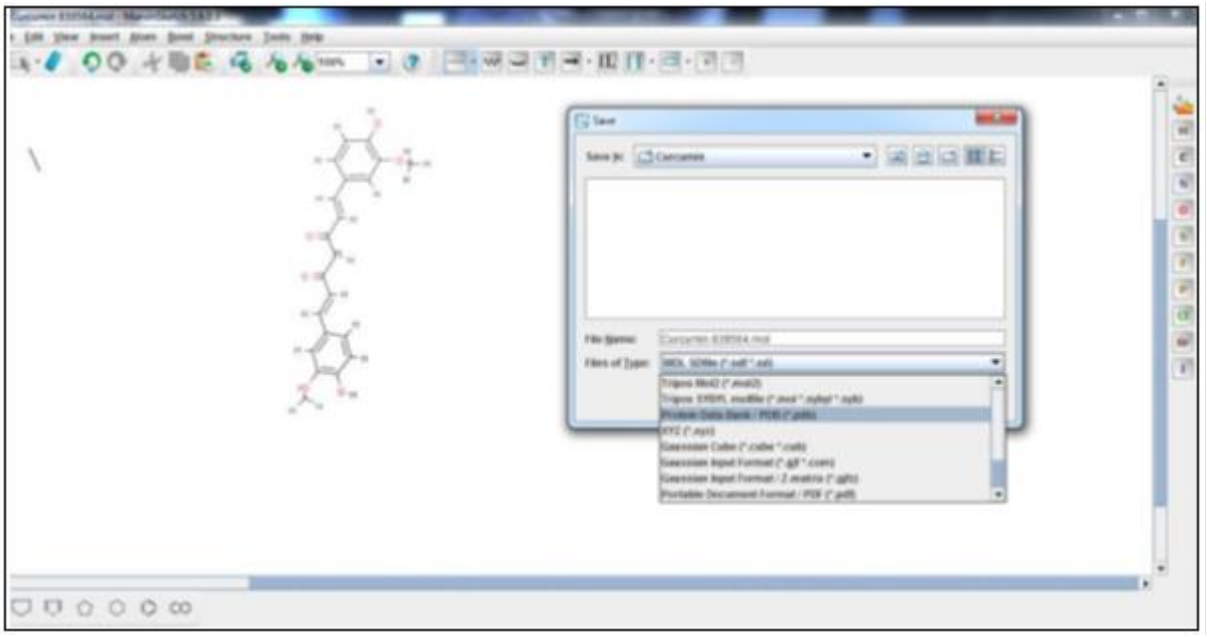


Figure 5 - Snapshot of Marvin sketch (conversion to .pdb)

6.4 Energy minimization of ligands:

Before binding energy calculations are performed, it is necessary to correct structural inconsistencies, add hydrogen, and associate atoms with force field parameters. Energy minimization is used to compute the equilibrium configuration of ligand. It makes the ligand flexible so that ligand can properly orient itself on the receptor during docking. Energy minimization was done by PRODRG, an online server following the given steps-

- Open Prodrg beta server.
- Paste the pdb format of ligand in the space provided & run Prodrg.
- Save the energy minimized pdb file (all hydrogens) generated by the server for future use.

Figure 6 - Snapshot of PRODRG server (online minimization page)

6.5 Energy minimization of Receptor:

The receptors obtained from the Protein Data Bank are generally complex files i.e., NMR structures of already docked receptor with some existing inhibitors. For using the same receptor for our docking studies, the receptor should be cleaned by eliminating the exiting ligand and the reactive groups created by removing this ligand should be substituted with hydrogens. For the removal of the ligands CHIMERA is used and from residue selecting option the Non amino acid groups can be selected and deleted. The resultant PDB structure can be saved. The following steps are followed-

- Double click chimera open receptor file
- Select all
- Tools --Structure editing -- minimize structure
- Choose the steps and minimise
- Save the resultant file in pdb format and use for docking

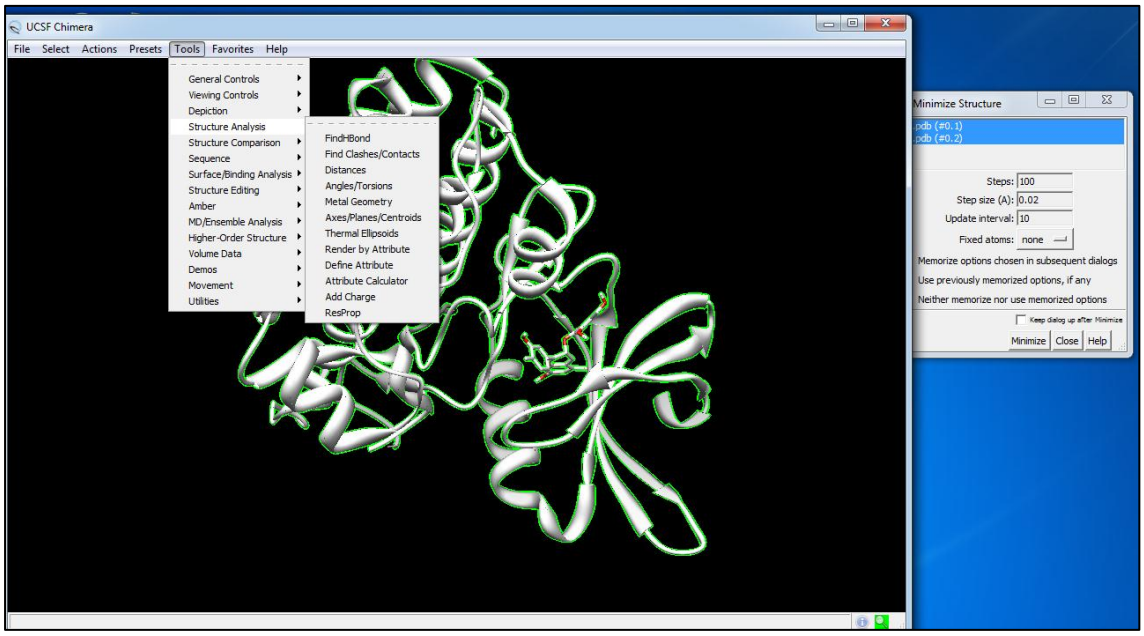


Figure 7 - Snapshot of CHIMERA (Protein energy minimization)

6.6 Docking Tools Autodock 4.0

Docking is a method which predicts the preferred orientation of a small molecule (ligand) on a larger molecule (receptor) when bound to each other to form a stable complex. Each docking program utilizes a unique scoring function to rapidly approximate properties such as receptor-ligand binding. Here Autodock is used as the docking tool which uses free-energy scoring function that is based on a linear regression analysis and the AMBER force field that occur between a ligand and protein target

The introduction of Autodock 4 comprises three major improvements:

- The docking results are more accurate and reliable.
- It can optionally model flexibility in the target macromolecule.
- It enables AutoDock's use in evaluating protein-protein interactions.

AutoDock 4 offers many new features and improvements over previous versions. The most significant is that it models flexible side chains in the protein. We can get both the 3D structure and the inhibition constants. AutoDock4 scoring functions are van der Waals forces, Hydrogen Bonding, Electrostatics, Desolvation, Torsional.

Binding energy=Intermolecular energy+ Torsional energy ΔGbind = ΔGvdw + ΔGele. + ΔGHβond + ΔG desolv +ΔGtors Here ΔG=change in free energy The aim of this part is to re-dock the ligand present in the crystal structure of a protein using an automated docking suite called 'AutoDock'. The GUI for AutoDock is AutoDockTools (ADT), which was used to perform the entire docking task. More information is available on the AutoDock suite homepage http://www.scripps.edu/mb/olson/doc/autodock/.After preparing the protein and ligand files through chimera, all files must be transferred to the Autodock directory. Further modification to the protein and ligand, something like fixing the torsion residues etc are made and the files are saved in PDBQT format. The grid box is setted on the protein. After Autodock completes, the docking results are saved in a file named dlg in the directory. The conformation with the lowest docking energy is ranked best by AutoDock. One can see the clusters of docked conformations based on Binding energies by Opening and scrolling down the dlg file until we find 'CLUSTERING HISTOGRAM'. Make a note of the cluster rank, lowest docked energy, number of conformations in the cluster.

AutoDock is used to perform computational molecular docking of small molecules to proteins, DNA, RNA and other important macromolecules, by treating the ligand and selected parts of the target as conformationally flexible. It uses a scoring function based on the AMBER force field, and estimates the free energy of binding of a ligand to its target. Novel hybrid global-local evolutionary algorithms are used to search the phase space of the ligand-macromolecule system.

The steps followed are as mentioned below-

- Get the receptor (1VJY) coordinates (i.e., from the PDB).
- Clean the receptor (delete all the water and all non-interacting ions).
- Add the missing hydrogens/side chain atoms and minimize the receptor
- Get the minimized ligand from PRODRG server
- Prepare the docking suitable files for LOCK and KEY (pdbqt files).
- Prepare all the needing files for docking (grid parameter file, map files, docking Parameter files).
- Run the docking for 100 runs.
- Get the docking results.

Grid Generation:

The Grid box was given position (78, 58, and 70) and grid centre (10.76, 73.1, and 5.436) xyz coordinates. The binding site includes active site (HIS283, LYS232, TYR249, GLU245, ASP351, SER280) being ATP binding site.

The Calculations of Auto grid and Autodock were performed on Linux operating system having system Properties (Intel(R) Pentium(R) D CPU 2.80GHz, 2.0 GB of RAM).

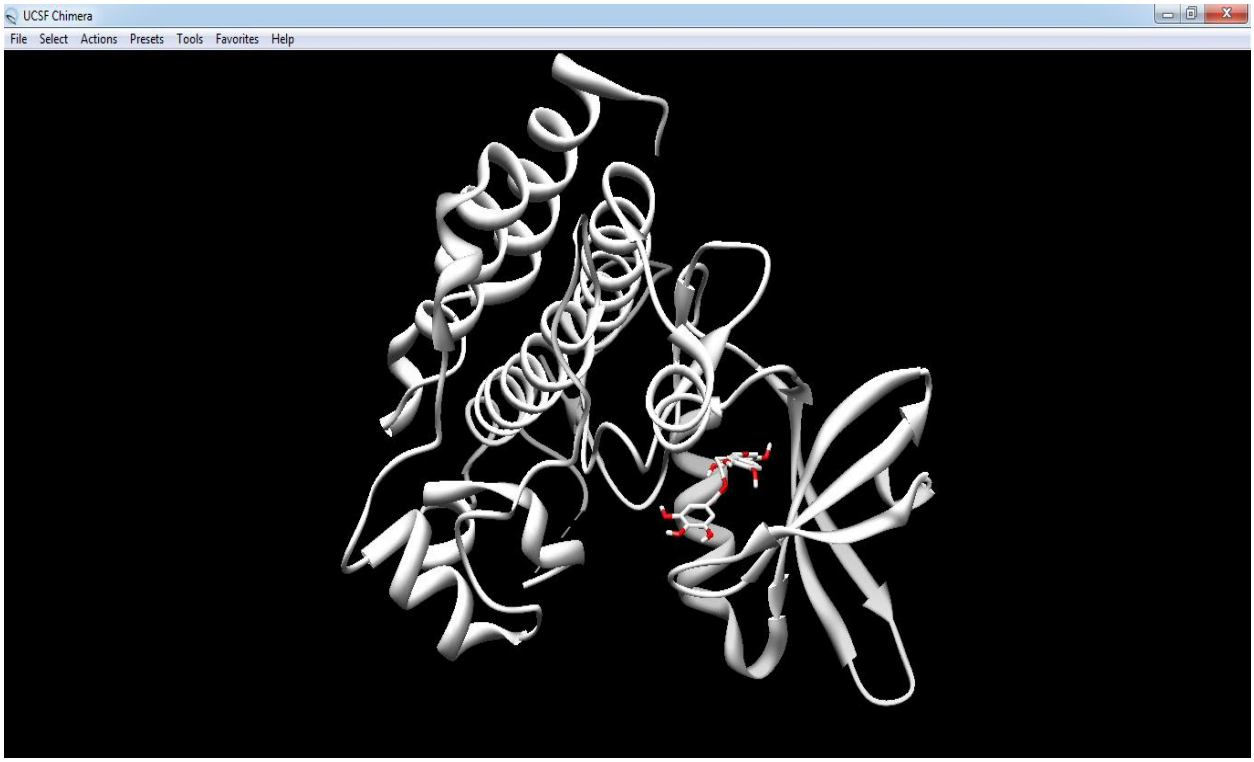


Figure 8 - Docked complex of TGFβ1 and Epicatechin Gallate

6.7 Analysing Docking Results:

Primarily the results of AutoDock can be analysed based on the best and lowest binding energies stored as a table in .dlg file (Docking Log file). This file can be opened using text editor and can be investigated for the inhibitory constant (Ki) and binding energy values. The interactions of the ligands with different amino acid residues of the receptor can found in detail by analysing the complex.pdb using

Ligand Scout.

The following steps are followed-

- Launch ligand Scout and open the complex.pdb
- Double click on the boxed region where the ligand is highlighted.
- Complex will be zoomed in to the interactive ligand-receptor region.
- Now go to pharmacophore menu and select normalized pharmacophore.
- The interactive amino acids of receptor with ligand are obtained.

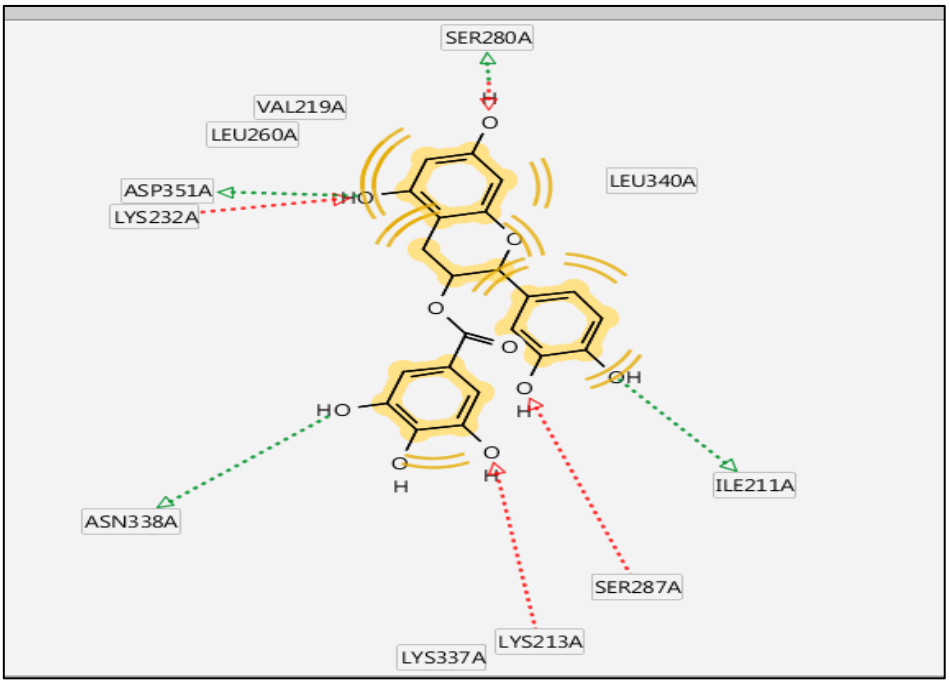


Figure 9 - Interacting amino acids with Epicatechin Gallate .

Ligplot

- Open complex file
- Change ligand ATOM to HETATM
- Carbon,nitrogen,oxygen, hydrogen numbering
- Replace numbering 1 2 3 with 5001 ,5002 like this
- Replace UNK with DRG
- Remove the data of docking in the complex file after receptor data

- Check the alignment with receptor alignment in the file
- Save the file in pdb format
- Open command promt
- Cd ligplot
- Ligplot filename.pdb DRG1 DRG1
- Open the .ps file in post script viewer for atomic interactions

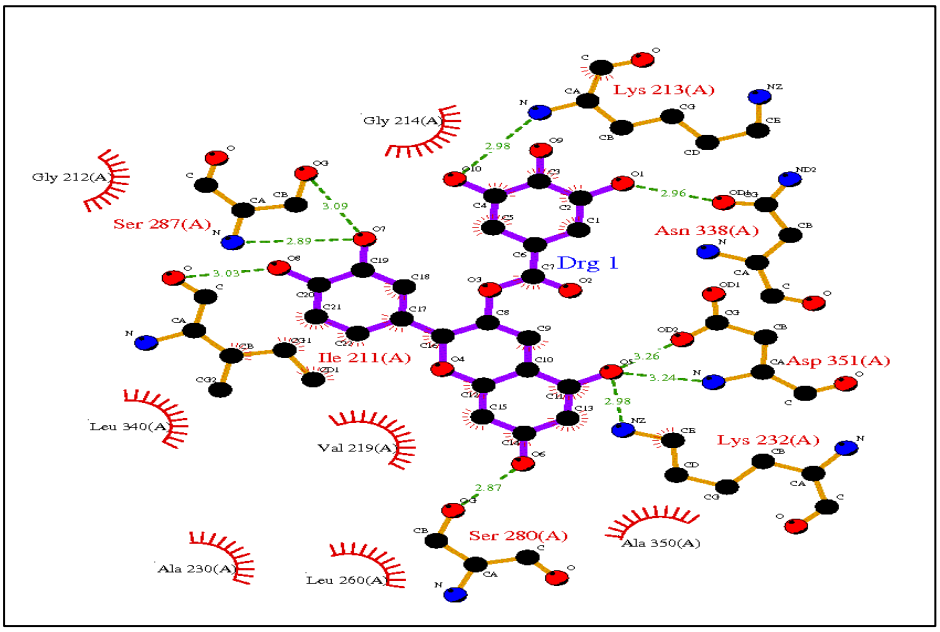


Figure 10 - Atomic interactions of TGFβ1 with Epicatechin Gallate

6.8 Structural variation of Epicatechin Gallate in designing new inhibitor for better binding

- Bioisosteric substitution the process of starting with an initial active compound and replacing functional groups with other groups that have similar biological properties.

  Ex - Monovalent groups H F Cl Br I OH SH NH_2 PH_2 CH_3 SCH_3 [48]
- Variation in substituents is a method of generating many similar compounds even though the rules of bioisosteric replacement may not be followed [49]

- An extension of the parent structure is usually carried out by adding functional groups in locations where there were none in the parent
- Extension and contractions of both chains and rings are another valuable set of experiments [50]
- Replacement of OH group with CH_3 for interaction with LYS 337A
- Replacement of OH group with CH_3 for interaction with VAL219A , LEU260A SER280A
- Replacement of OH group with CH_3 for interaction with ILE211A

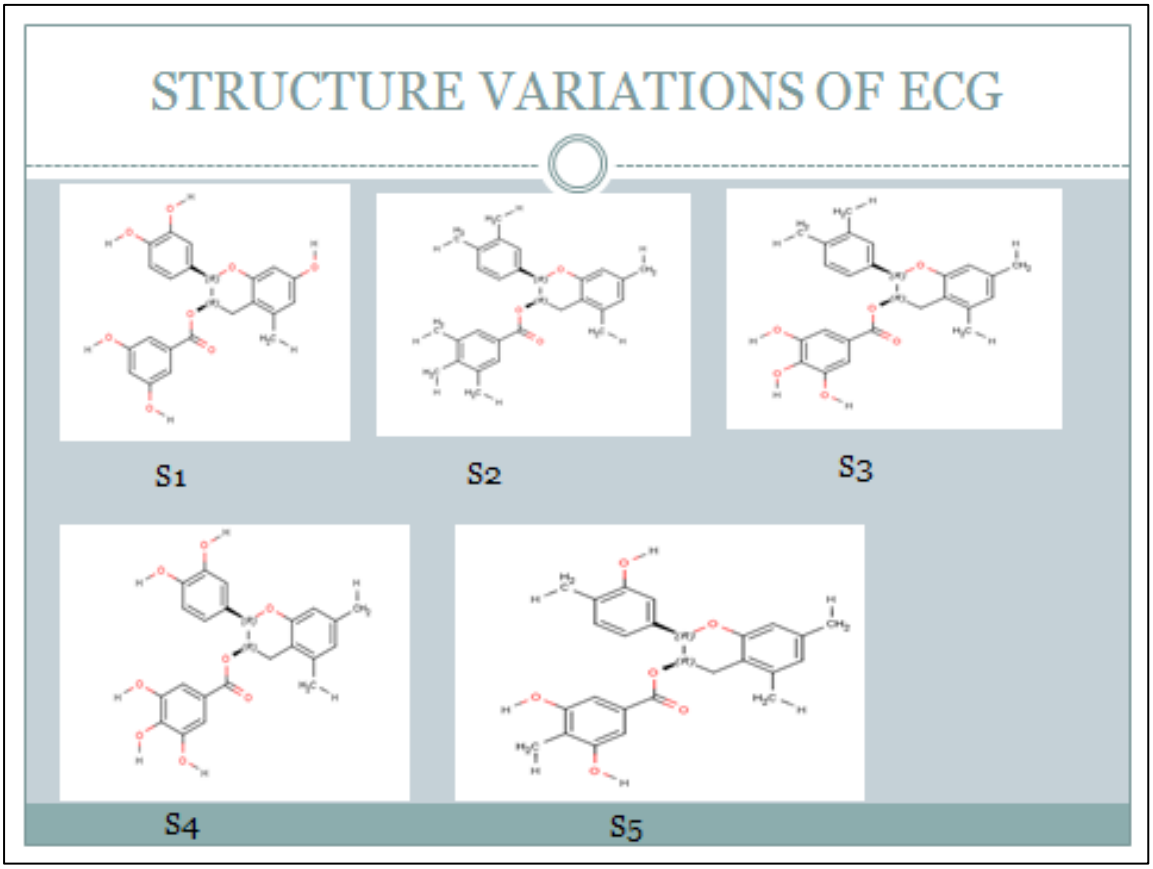


Figure 11- Structural variations of Epicatechin Gallate

6.9 Pre ADMET Studies:

The success of a drug depend on its ADME (Absorption, Distribution, Metabolism and Elimination) characters in the body. The early stage prediction of ADME of a new ligand reduces the probability of its failure at the drug development stage. Thus, these studies help in the Insilico analysis of not only ADME prediction but Molecular descriptors calculation, Drug likeness prediction, Drug toxicity prediction. The steps involved in Pre ADMET studies are as follows-

- Open Pre ADMET server (http://preadmet.bmdrc.org/)
- Register and login to the server
- Prepare the .mol file of the ligands for analysis
- Analyze the Drug likeness, ADME and Toxicity of the ligand from different tabs available in the login.

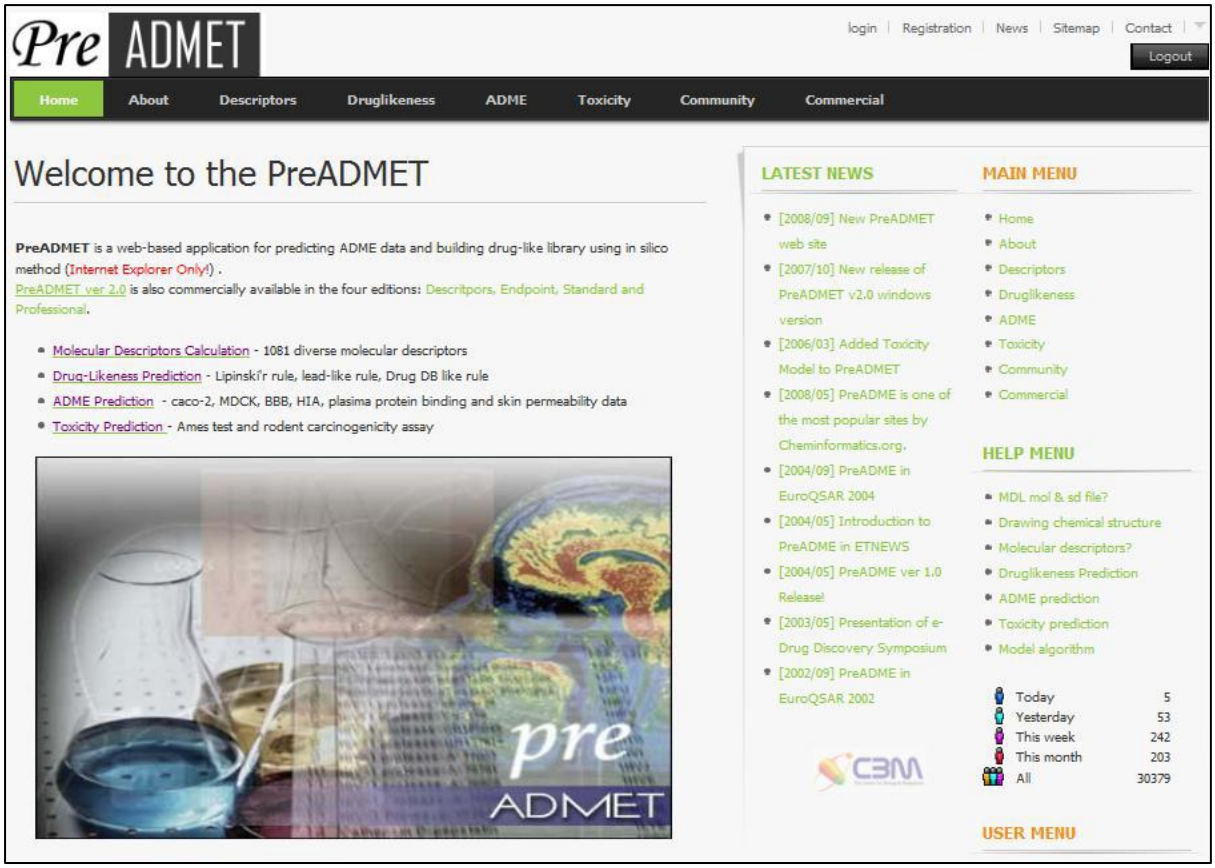


Figure 12 - Pre ADMET server home page

6.10 MOLECULAR DYNAMICS SIMULATION by CHARMm:

The molecular dynamics of the protein–inhibitor complex provides understanding to the flexibility associated with ligand conformational change, and thus provides an insight into molecular basis for inhibition. All of the simulations were carried out using the CHARMm package with an identical protocol [51]. The best orientation obtained out of docking of protein-ligand complex was used for simulation. We performed simulation for ligands SB505124 and S1and their respective binding with TGF-β. Here we separated the ligands from protein in order to prepare protein and ligand topology file separately. We used CHARMm force filed to generate topology file for protein. . The protein was solvated in a orthorhombic box with edges 1 nm in length using the explicit solvent–simple point charge model, which generated the water box. The next step followed the 2000 steps of steepest descent minimization and position restrained dynamics to distribute water molecule

throughout in 1000 ps. The next step of minimization followed conjugate gradient of 20000 steps. The simulation was carried out at 300k of constant temperature and pressure of 500000 steps for 1ns. Once the system was equilibrated with desired temperature and pressure the final step was to release the position restrains and run production of 125000 steps for 1ns for data collection.

7. RESULT AND DISCUSSION

7.1 Results of existing inhibitors docking:

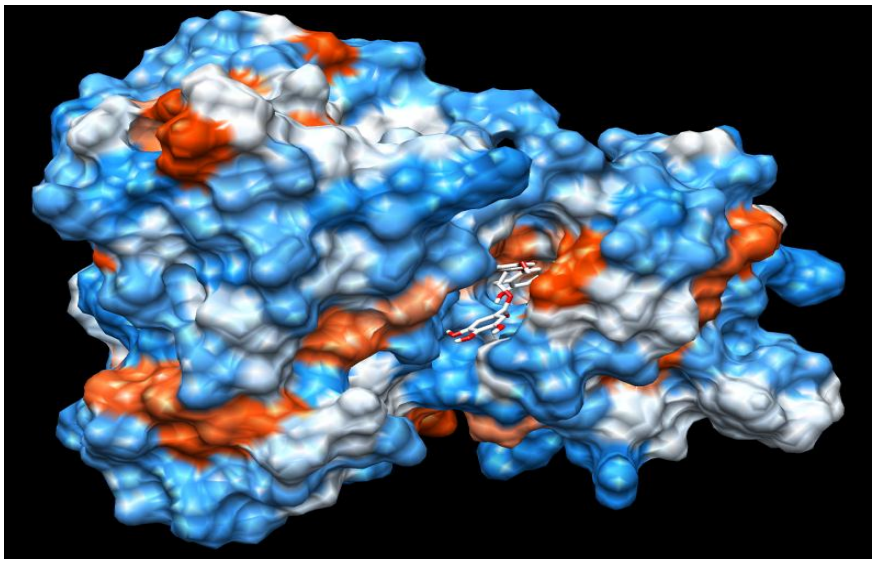


Figure 13 - Docked complex of TGFβR1 and Epicatechin Gallate.

Drug bank is the source of newly synthesized molecules with unknown activity. We have downloaded a group of small inhibitors from drug bank and selected few from literature for docking with TGFβR1. The results are tabulated below-

INHIBITOR	**BINDING ENERGY (-VE)**	**Kı(μM)**
CID_447966	7.74	2.11
CID_11676119	9.40	0.112
CID_11599374	8.12	0.0112
CID_23507473	8.68	0.434
CID_49788486	8.74	0.390
CID_25138294	8.19	0.996
CID_23002763	4.50	501.8
SB-431542	8.53	0.402
SB505124	**9.18**	**0.186**
EW7204	9.19	0.183
CID_107905(Epicatechin Gallate)	**9.44**	**0.237**
CID_65064(EGCG)	9.04	0.120
SM16	9.17	0.190
SD208	8.0	1.37
LY5504	8.76	0.382
LY580276	7.55	2.93

Table.1 Docking results of 16 Drug bank molecules with TGFβR1

The Ligands and the receptors were retrieved and minimized as a preparatory step for Docking. Molecular Docking is performed using AutoDock 4. The TGFβR1 receptor is docked with the Epicatechin Gallate ligand which gave a good binding energy -9.44 k.cal/mol. These results are compared with a standard SB505124 which gave a binding energy of -9.18 k.cal/mol, but Epicatechin Gallate cannot be used for severe scar hence further investigation is required to find out the effective inhibitor.

7.2 Results of newly designed inhibitors docking:

INHIBITOR	BINDING ENERGY (-VE)	Kı(μM)
S1	10.47	0.021
S2	10.16	0.035
S3	9.83	0.062
S4	9.98	0.048
S5	10.48	0.020

Table.2 Docking results of 5 newly designed inhibitors with TGFβR1

Newly designed inhibitor s5 and s1 showed better binding energy than existing inhibitor

7.3 Amino acids interactions with the inhibitors

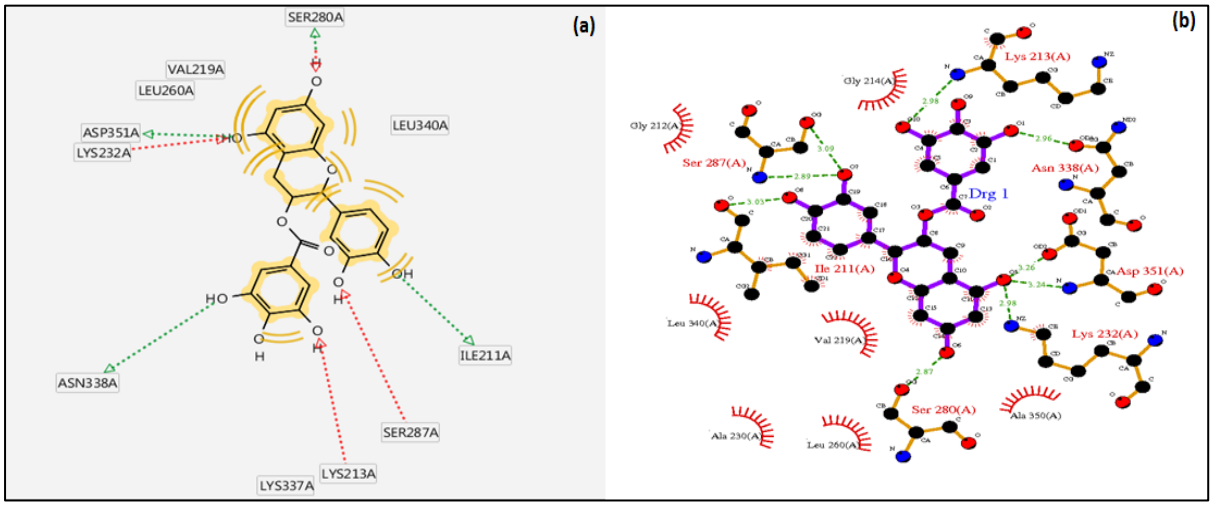


Figure 14 - Inhibitor Epicatechin Gallate interacting with amino acids from ligand scout and ligplot.

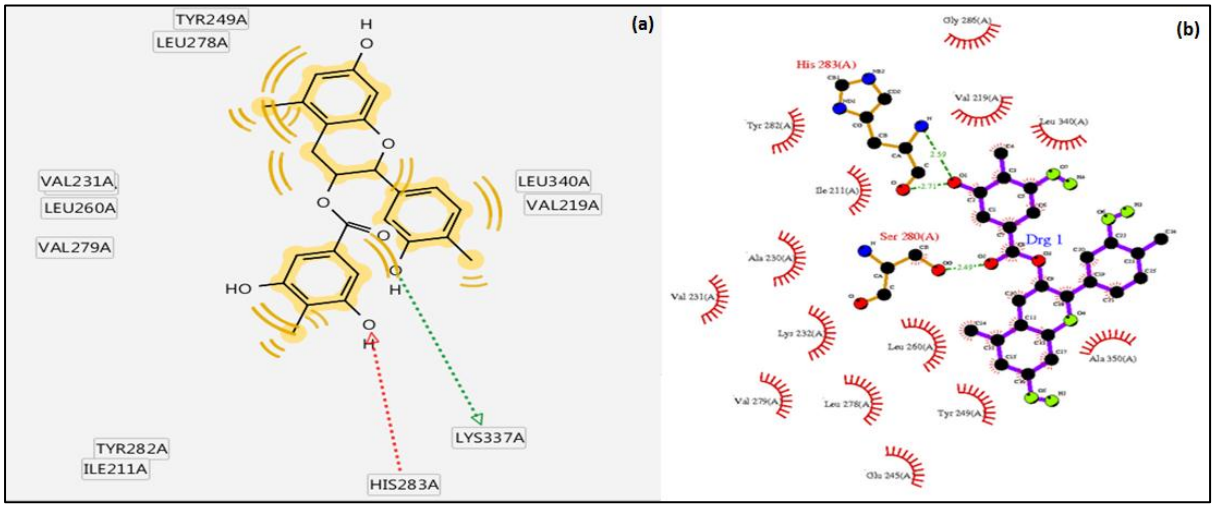


Figure 15 - Interactions of S5 inhibitor with TGFβR1 amino acids (a), Atomic interactions with Amino acids (b).

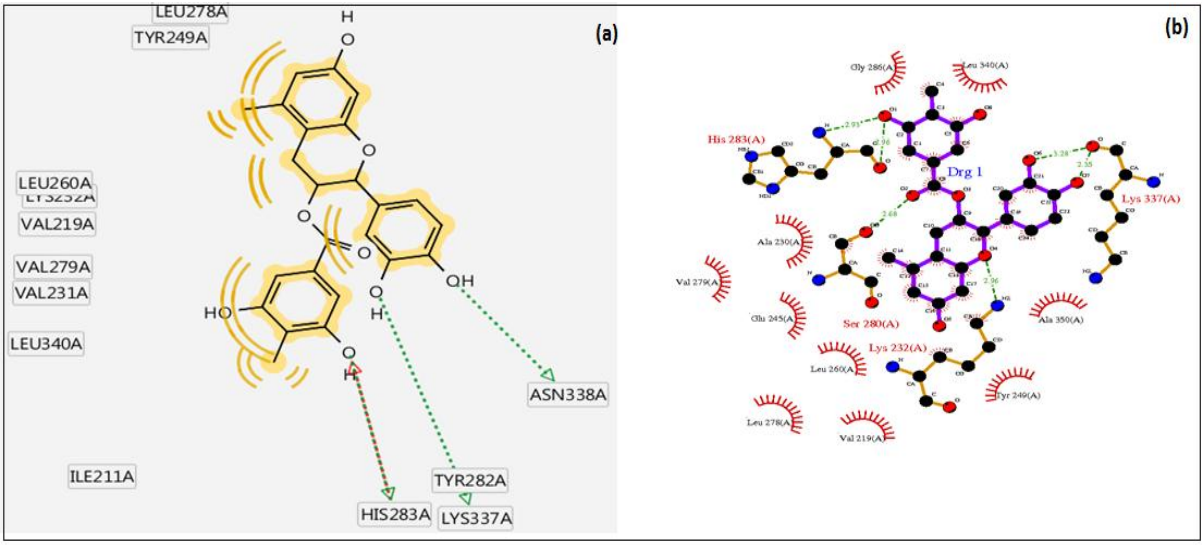


Figure 16 - Interactions of s1 inhibitor with TGFβR1 amino acids (a), Atomic interactions with Amino acids (b).

Figure 15 & 16 clearly shows many hydrophobic interactions hence giving better binding energy.

7.4 PreADMET Results:

Based on the binding energy of the existing inhibitors and newly designed inhibitors with TGFβR1 and the Ki value of the best conformation, are selected for PreADMET studies to know their Molecular descriptions, Drug Likeness, ADME data, Toxicity. Out of five newly designed inhibitors only s1 qualified as drug. The results are tabulated as follows-

INHIBITOR	Rule's qualified	Human intestinal Absorption (%)	Invitro skin permeability (logKp, cm/hour)	Toxicity
ECG	CMC like rule	40.58	-3.88	Non mutagen Non carcinogenic (mouse)
SB505124	Lipinski rule, CMC like rule, WDI rule	92.98	-3.23	Non mutagen Non carcinogenic (mouse)
S1	Lipinski rule, CMC like rule, WDI rule	79.29	-3.61	Non mutagen Non carcinogenic (mouse)
S2	-	98.97	-1.88	Non mutagen Non carcinogenic
S3	-	92.74	-2.40	Non mutagen Non carcinogenic (mouse)
S4	CMC like rule, WDI rule	80.35	-3.56	Non mutagen Non carcinogenic (mouse)
S5	-	92.74	-2.39	Non mutagen Non carcinogenic (mouse)

Table 3- Preadmet results of newly designed inhibitors

S1 is found non toxic and qualified all the rule having good skin permeability ,hence can be used as effective drug but further its stability has to be verified with molecular dynamic simulation studies.

7.5 Molecular dynamic simulation

In Molecular Dynamic Simulation, energy calculation plays a vital role to determine the stability of the complex during simulation. Variation in kinetic energy, total energy components and potential energy verses time in MD Simulation are shown in Figure-17 and 18 for Epicatechin Gallate, S1 respectively. From the plots it is clear that the kinetic energy and potential energy components fluctuate in equal and opposite direction. For the stability of the system, conservation of energy should be maintained. The figures show that conservation of energy is satisfied during MD Simulation, which indicates that the complexes during

simulation are quiet stable. Graph of all the two complexes shows that the kinetic energy is highest and the potential energy is lowest and this condition is required for a stable system.

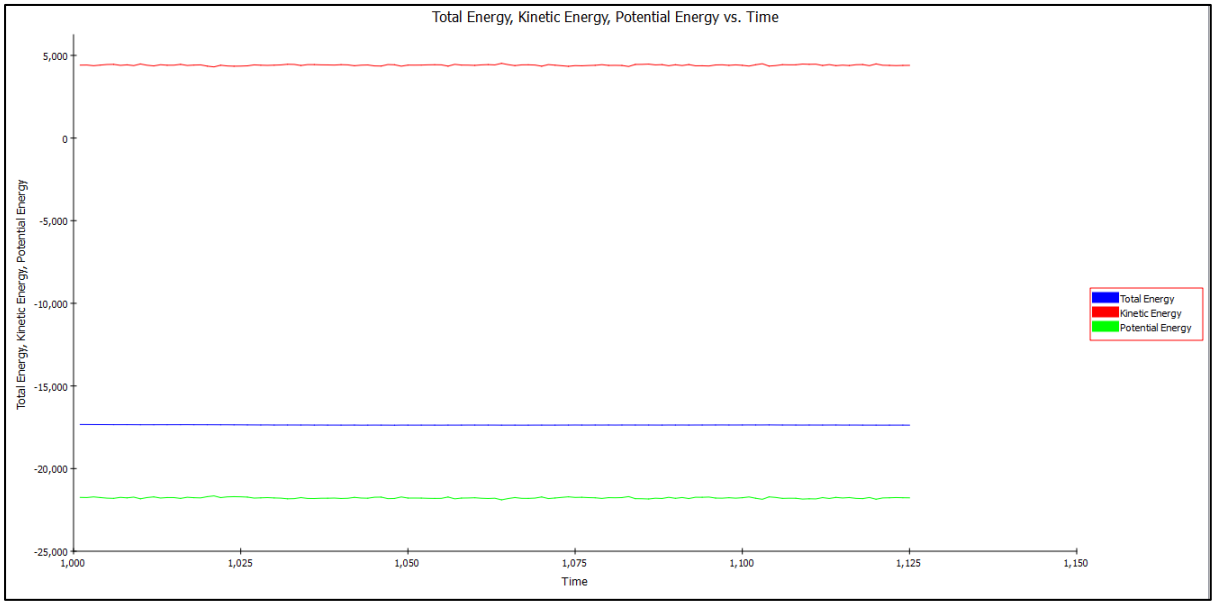


Figure 17- Energy diagram of ECG complex

The energy diagram of the ECG and TGFβR1 complex is shown here. It shows that the kinetic energy of the system is maximum and it is about 5000 $KJmol^{-1}$. Potential energy is minimum and it is of -20000 $KJmol^{-1}$. Total energy is -17000 $KJmol^{-1}$ and the value is in between K.E and P.E. the system is in stable condition since potential energy is negative and least among all the energy value. And also conservation of energy is followed here. So, ECG is showing its stability during the course of molecular dynamic simulation.

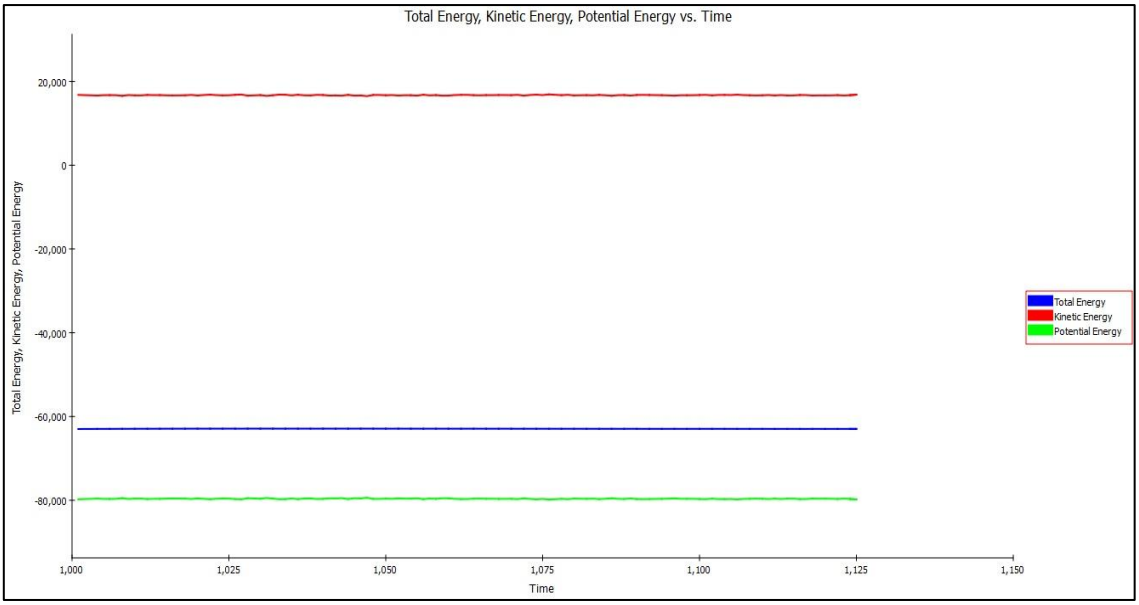


Figure 18 - Energy diagram of S1complex

The energy diagram of the S1 is shown here. It shows that the kinetic energy of the system is maximum and it is about 20000 KJmol-1. Potential energy is minimum and it is of -80000 KJmol-1. Total energy is -60000 KJmol-1 and the value is in between K.E and P.E. the system is in stable condition since potential energy is negative and least among all the energy value. And also conservation of energy is followed here. So S1 is showing its stability during the course of molecular dynamic simulation

RMSD

RMSD (Root Mean Square Deviation) is used to measure the conformational stability of a complex during simulation. The RMSD of the backbone atoms of the protein from the starting structure over the course of simulation may be used to measure the conformational stability of a protein during that simulation. The RMSD of the complex versus time plot are shown in the following figures. The dynamic behaviour and structural change of the receptor was evaluated by calculating the RMSD value. ECG Complex fluctuates between the RMSD values of 0.25Å-0.50Å as shown in figure19, whereas S1 complex shows less fluctuation

RMSD value between 0.25Å to 0.27Å as shown in figure 20. All the complexes showed RMSD value in the highly acceptable range, with very less fluctuation. The outcome results indicate that the complexes formed by these ligands and receptors are quiet stable.

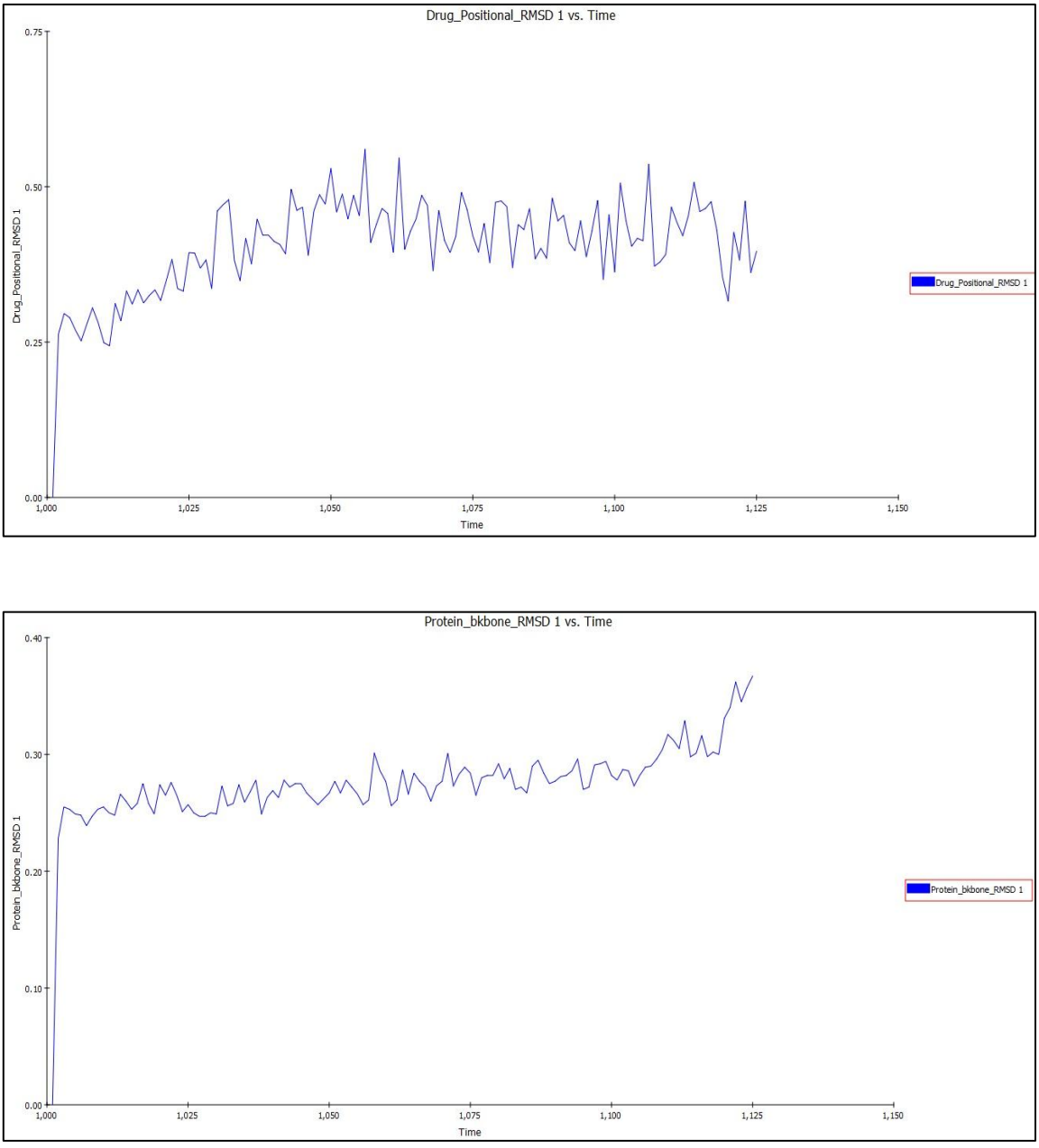


Figure 19 - RMSD plot cof ECG complex

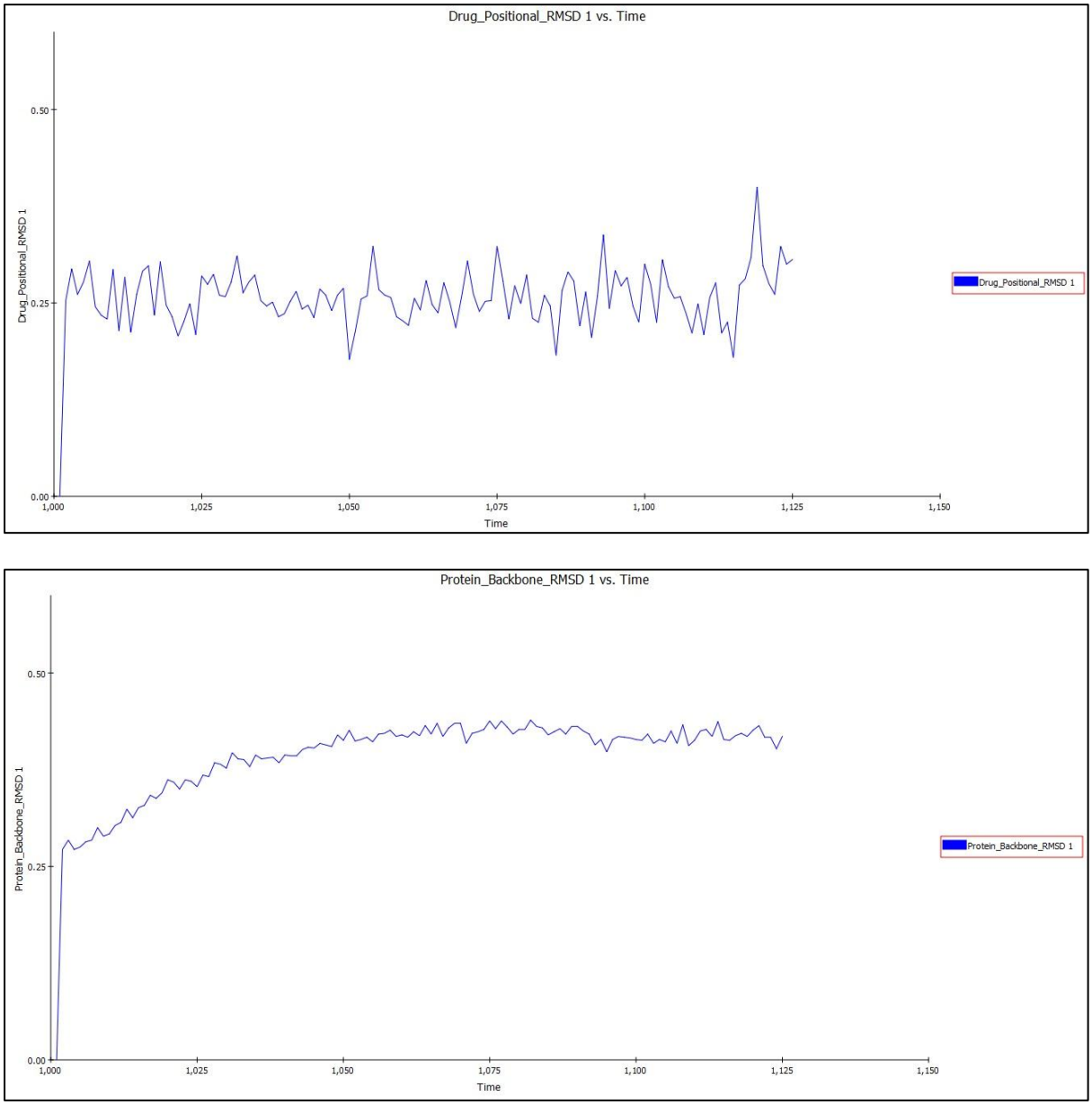


Figure 20 – RMSD plot of S1 complex

MD simulations were performed to determine the stability of the protein-ligand complex. The total energy drift during entire production simulation of the second 1000 ps, the drift was −17372,-62957 (kcal/mol) for respective protein ligand complex. The lower the value for total energy more stable is the complex and higher is the binding affinity. Hence S1 having less energy valve is showing better binding affinity than ECG. Both the complex were stable at body temperature of 300k To monitor the stability of the RMSD values of all the atoms of

protein-ligand complex to relative to the starting structure versus simulation time were measured. As can be seen from the plot, the RMSD fluctuations are similar in both the systems around 0.30 Å. Both the complexes were likely to reach equilibrium at 1ns. Further ahead the drug positional rmsd was used to determine the mobility of the drug in protein binding pocket. The ligands exhibited root mean square deviation of 0.50, 0.25 Å respectively as shown in figure 19 &20. The lower deviation exhibit the ligand to be closer to protein binding site forming stable complex. Thus from the above results the conclusion can be drawn is that the S1 complex was comparatively more stable as compared to parent ECG complex.

8. CONCLUSION

By analysing the Molecular Docking results i.e., binding energies of all the molecules Epicatechin Gallate possesses promising inhibitory activity against TGFβ1 receptor. But this inhibitor won't be effective in case of severe scar hence to further strengthen the binding affinity; five new ligands were designed by structural modifications of Epicatechin Gallate. Out of five derivatives of Epicatechin Gallate, S1 & S5 showed better binding than existing inhibitors. Further the Pre ADMET results showed a positive outcome with S1 compound showing good dermal absorption, drug likeness and non-carcinogenicity. Its stability was validated by MD Simulation at 1ns, RMSD, and Energy graphs showed high stability of the ligand-receptor complex at the time of Molecular Dynamic Simulation. Combining the above outcomes, we can conclude that the S1 as effective inhibitor of TGF-β pathway can be used in the superficial severe scar treatment. However, the results need further confirmation by wet lab experiments followed by clinical trials before it's translation into clinics.

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